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# Suppression of VEGF-mediated autocrine and paracrine interactions between prostate cancer cells and vascular endothelial cells by soy isoflavones☆,☆☆

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**Abstract**

Angiogenesis is an essential process involved in the development and progression of prostate cancer. Vascular endothelial growth factor (VEGF) is hypothesized to be a critical regulator of angiogenesis during prostate carcinogenesis. We have reported that dietary soy products inhibit prostate tumor progression in animal models, in association with a reduction in tumor microvessel density. The goal of the present study is to investigate potential antiangiogenic mechanisms of genistein, the major soy isoflavone, using in vitro systems. Genistein (5–50 μM) significantly inhibited the growth of human umbilical vein endothelial cells (HUVECs) in control media when stimulated by supplemental VEGF or when cultured in hypoxia-exposed PC-3 prostate adenocarcinoma cell conditioned media. These in vitro studies suggest detectable inhibitory effects by 5–10 μM genistein ($P<.05$) with an $IC_{50}$ of approximately 20 μM or less. Genistein (10–50 μM) caused significant inhibition of basal VEGF expression and hypoxia-stimulated VEGF expression in both human prostate cancer PC-3 cells and HUVECs based on semiquantitative reverse transcription–polymerase chain reaction ($P<.05$). In parallel, VEGF secretion by PC-3 cells quantitated by enzyme-linked immunosorbent assay was significantly ($P<.05$) reduced by genistein (10–50 μM). Furthermore, genistein (10–50 μM) significantly ($P<.05$) reduced PC-3 nuclear accumulation of hypoxia-inducible factor-1α, the principle transcription factor that regulates VEGF expression in response to hypoxia. Expression of the VEGF receptor fms-like tyrosine kinase-1, but not kinase insert domain-containing kinase, in HUVECs was also reduced ($P<.05$) by genistein (10–50 μM). These observations support the hypothesis that genistein may inhibit prostate tumor angiogenesis through the suppression of VEGF-mediated autocrine and paracrine signaling pathways between tumor cells and vascular endothelial cells.


*Keywords:* Genistein; VEGF; HIF-1α; Prostate cancer; HUVEC

*Abbreviations:* FLT-1, fms-like tyrosine kinase-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF-1α, hypoxia-inducible factor-1α; HUVEC, human umbilical vein endothelial cell; KDR, kinase insert domain-containing region; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PI3K, phosphatidylinositol 3′-kinase; VEGF, vascular endothelial growth factor.

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## 1. Introduction

Tumor angiogenesis is a complex biological process involving a dynamic interaction between cancer cells and those of the host microenvironment and is considered essential for tumor development and progression [1,2]. An array of hormones, growth factors and cytokines orchestrates tumor-associated angiogenesis by shifting the homeostatic balance in the microenvironment towards vessel formation [3,4]. Vascular endothelial growth factor (VEGF), originally described as a vascular permeability factor, has been implicated as one of the most important proangiogenic growth factors during carcinogenesis [5]. VEGF stimulates endothelial cell proliferation and differentiation, leading to primitive vessel formation [6,7]. VEGF is known to elicit its biological roles through interaction with two classes of VEGF receptors, fms-like tyrosine kinase-1 (FLT-1) and kinase insert domain-containing region (KDR), which are found in vascular endothelial cells and propagate signals through receptor-associated protein tyrosine kinases [8,9]. The transcription factor hypoxia-inducible factor-1α (HIF-1α) is one of the most important regulators of VEGF gene expression in response to hypoxia in tumor cells [7,10]. Reduced oxygen tension is characteristic of the tumor microenvironment due to imbalance between accumulating tumor cells and lagging angiogenic responses. This imbalance contributes to sluggish blood flow caused by an irregular and poorly formed vasculature [11]. HIF-1α protein is constitutively synthesized and continuously degraded under normoxic conditions. However, the cellular content of HIF-1α is dramatically increased by hypoxia through a combination of increased synthesis and decreased degradation, thereby contributing to increased VEGF expression [9,12,13]. In addition to the paracrine stimulation of endothelial cells by VEGF secreted from tumor cells, vascular cells may also produce VEGF and thus stimulate angiogenesis through an autocrine fashion [14].

Accumulated evidence suggests that VEGF-stimulated angiogenesis is a component of human and experimental prostate carcinogenesis. Increased vascular density has been documented in prostatic intraepithelial neoplasia (PIN) relative to benign epithelium and is greater in locally advanced cancers or metastasis compared with organ-confined tumors [15]. Similarly, vascular density is increased during rodent prostate carcinogenesis and is associated with areas within tumors that show increased proliferation [16]. Several studies suggest that VEGF expression is enhanced in tumor cells, vascular endothelial cells and stromal cells within a developing prostate cancer, whereas normal prostate tissues show lower expression [17]. HIF-1α immunohistochemical (IHC) staining is significantly increased in high-grade PIN lesions compared with normal epithelium, stromal cells and benign prostatic hyperplasia tissues, which is further enhanced in prostate cancer lesions [18]. In addition, increased plasma VEGF concentrations are found in prostate cancer patients compared with healthy controls [19]. Plasma VEGF levels are increased with the progression of prostate cancer to locally advanced and metastatic disease and with the development of androgen independence [19,20]. Consequently, anti-VEGF therapy is now being evaluated in prostate cancer clinical trials [21].

Our laboratory is evaluating angiogenesis as a target for dietary or chemopreventive interventions that may inhibit prostate carcinogenesis. For example, we have observed that dietary restriction reduces prostate tumor growth in association with reduced vascular density and lower VEGF expression in the tumor microenvironment [22]. We have also shown that diets containing soy protein or a phytochemical-rich extract also inhibited prostate tumor growth in murine transplantable models, in parallel with reduced intratumor vascularity [23].

In the present in vitro study, we examine the ability of genistein to act upon specific aspects of VEGF-mediated autocrine and paracrine interactions between prostate tumor cells and vascular endothelial cells. We observed that genistein inhibited endothelial cell proliferation and tube formation in an angiogenic assay, reduced hypoxia-induced VEGF expression in both prostate cancer and endothelial cells, reduced VEGF receptor expression in endothelial cells and decreased HIF-1α expression in prostate cancer cells in response to hypoxia. These studies establish mechanisms whereby soy isoflavones may impact interactions between tumor cells and the host microenvironment, which can be evaluated in future rodent or human translational studies.

## 2. Materials and methods

### 2.1. Cell culture

Androgen-insensitive PC-3 human prostate cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained as monolayer cultures in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20,000 IU/L penicillin and 20 mg/L streptomycin. Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA) and maintained as monolayer cultures in an endothelial cell growth medium (EGM-2; Clonetics) supplemented with hydrocortisone, human epidermal growth factor, VEGF, insulin-like growth factor-1, fibroblast growth factor, heparin, ascorbic acid, gentamicin and 1% FBS. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% $CO_2$. For hypoxia treatments, cells were incubated in a sterile chamber flushed with 1% $O_2$, 5% $CO_2$ and 94% $N_2$ or incubated with 100 μM $CoCl_2$.

### 2.2. Cell growth assay

HUVECs ($4\times10^3$ per well) were seeded in 96-well tissue culture plates and incubated overnight. Cells were then treated with vehicle or genistein (Sigma, St. Louis, MO) in the presence or absence of 10 ng/ml VEGF (R&D Systems,

Minneapolis, MN) for 72 h, 24-h normoxia-exposed PC-3 conditioned media or 24-h hypoxia-exposed PC-3 conditioned media for 48 h. Cell proliferation was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay (CellTiter 96 $AQ_{ueous}$ One, Promega, Madison, WI). In brief, the MTS assay is an indirect measurement of viable cells based on the ability of dehydrogenase enzymes in metabolically active cells to cleave the tetrazolium salt MTS and to yield a highly colored water-soluble formazan product. The addition of the electron-coupling agent phenazine ethosulfate potentiates colorimetric reaction. All assays were completed in triplicate, and results were confirmed by direct cell counting with a hemocytometer.

### 2.3. In vitro tube formation assay

An in vitro tube formation assay with HUVECs was performed with the In Vitro Angiogenesis Assay Kit (Chemicon International, Temecula, CA). Briefly, 50 μl of cold ECMatrix solution was added to assigned wells of 96-well tissue culture plates and then incubated for 1 h at 37°C to solidify. Cells ($5\times10^3$) were then seeded onto the ECMatrix surface and treated with vehicle or genistein in the presence or absence of conditioned media from PC-3 cells with 24-h exposure to hypoxia. After overnight incubation at 37°C, tube formation was visualized by an Olympus IMT2 light microscope (×10), and images were captured with an Olympus SC35 camera (Olympus America, Inc., Melville, NY). Tube formation was quantitated by counting the number of branching points on each image [24].

### 2.4. Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis

RNA was extracted with the Absolutely RNA isolation kit (Stratagene, La Jolla, CA). RT-PCR was performed with 1 μg of total cellular RNA and the RiboClone cDNA Synthesis Kit (Promega). The following primers were used: (a) human VEGF primers: sense (5′-CGA AGT GGT GAA GTT CAT GGA TG-3′ ) and antisense (5′-TTC TGT ATC AGT CTT TCC TGG TGAG-3′ ); (b) human FLT-1 primers: sense (5′-CACAGTCCGGCACGTAGGTGATT-3′ ) and antisense (5′-GTCACAGAAGAGGATGAAGGTGTCTA-3′ ); (c) human KDR primers: sense (5′-CTGGCATGGTCT-TCTGTGAAGCA-3′ ) and antisense (5′-AATACCAGTG-GATGTGATGCGG-3′ ); (d) human HIF-1α primers: sense (5′-CTC AAA GTCGGACAGCCT CA-3′ ) and antisense (5′-CCC TGC AGT AGG TTT CTG CT-3′ ); and (e) human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Clontech, Palo Alto, CA): sense (5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′ ) and antisense (5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′ ). The conditions employed for RT-PCR were as follows: 95°C for 45 s, 60°C for 2 min and 72°C for 2 min (25–28 cycles), and final extension at 72°C for 7 min using a DNA thermal cycler (MJ Research, Inc., Alameda, CA). Amplified RT-PCR products were analyzed by electrophoresis (1% agarose gel), visualized by ethidium bromide staining and photographed under UV illumination. RT-PCR results were photographed and evaluated by densitometry (Alpha Innotech Corporation, San Leandro, CA). Relative gene expression levels were expressed as the percentage of the corresponding GAPDH control.

### 2.5. Western blot analysis

PC-3 cells were treated with 0, 10 and 50 μM genistein for 24 h then further incubated under normoxic conditions, under hypoxic conditions or with $CoCl_2$ (100 μM) for 24 h. Cells were harvested and nuclear protein was extracted with NE-PER Nuclear Extraction Reagents following the manufacturer's protocol (Pierce Biotechnology, Rockford, IL). Total protein concentration was determined with the DC

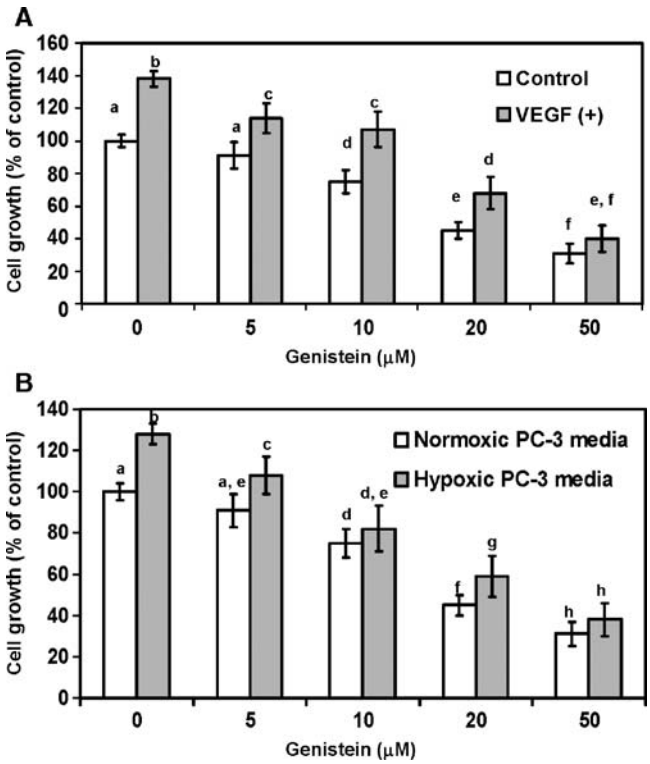


Fig. 1. (A) Genistein inhibited HUVEC growth with or without VEGF stimulation. HUVECs were treated with increasing concentrations of genistein in the presence or absence of VEGF (10 ng/ml) for 72 h. The cell viability of HUVECs was evaluated by MTS assay ($n=9$) and subsequently confirmed by direct cell counting (data not shown). VEGF significantly stimulated HUVEC growth, while genistein inhibited cell growth with or without additional VEGF stimulation. (B) Genistein inhibited the proliferation of HUVECs incubated with conditioned media from PC-3 cells grown under normoxic or hypoxic conditions for 24 h. HUVECs were seeded in 96-well plates and treated with conditioned media in the presence or absence of genistein for 48 h. HUVEC viability was evaluated by MTS assay ($n=9$) and subsequently confirmed by direct cell counting (data not shown). HUVEC growth in conditioned media from PC-3 cells grown under control conditions is considered 100%. Genistein elicited the growth inhibition of HUVECs incubated with conditioned media. Data are presented as mean±S.D. Means with different letters indicate a statistically significant difference ($P<.05$, by ANOVA and pairwise comparison).

Protein Assay (Bio-Rad, Hercules, CA). An equal amount of protein was loaded into each well, separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were incubated with monoclonal mouse anti-human HIF-1α (Novus Biologicals, Inc., Littleton, CO) at 1 μg/ml or with polyclonal rabbit antihuman actin (Sigma) at a 1:1000 dilution for 1 h at room temperature. The membranes were subsequently incubated with appropriate HRP-linked secondary antibodies for 30 min, and immunoreactive proteins were detected by enhanced chemiluminescence (Santa Cruz Biotech., Santa Cruz, CA) and exposure to film. Films were scanned and bands were quantified by densitometry (AlphaImager 2000; Alpha Innotech Corporation). Relative expression levels were expressed as the percentage of actin levels.

### 2.6. VEGF enzyme-linked immunosorbent assay (ELISA)

PC-3 cells ($2\times10^5$) were seeded with complete media in six-well tissue culture plates and incubated overnight. Cells were further incubated with a designated medium for an additional 48 h. Culture medium was collected, and VEGF concentrations were measured with the human VEGF Quantikine ELISA (R&D Systems), according to the manufacturer's protocols. VEGF concentrations were adjusted to cell number.

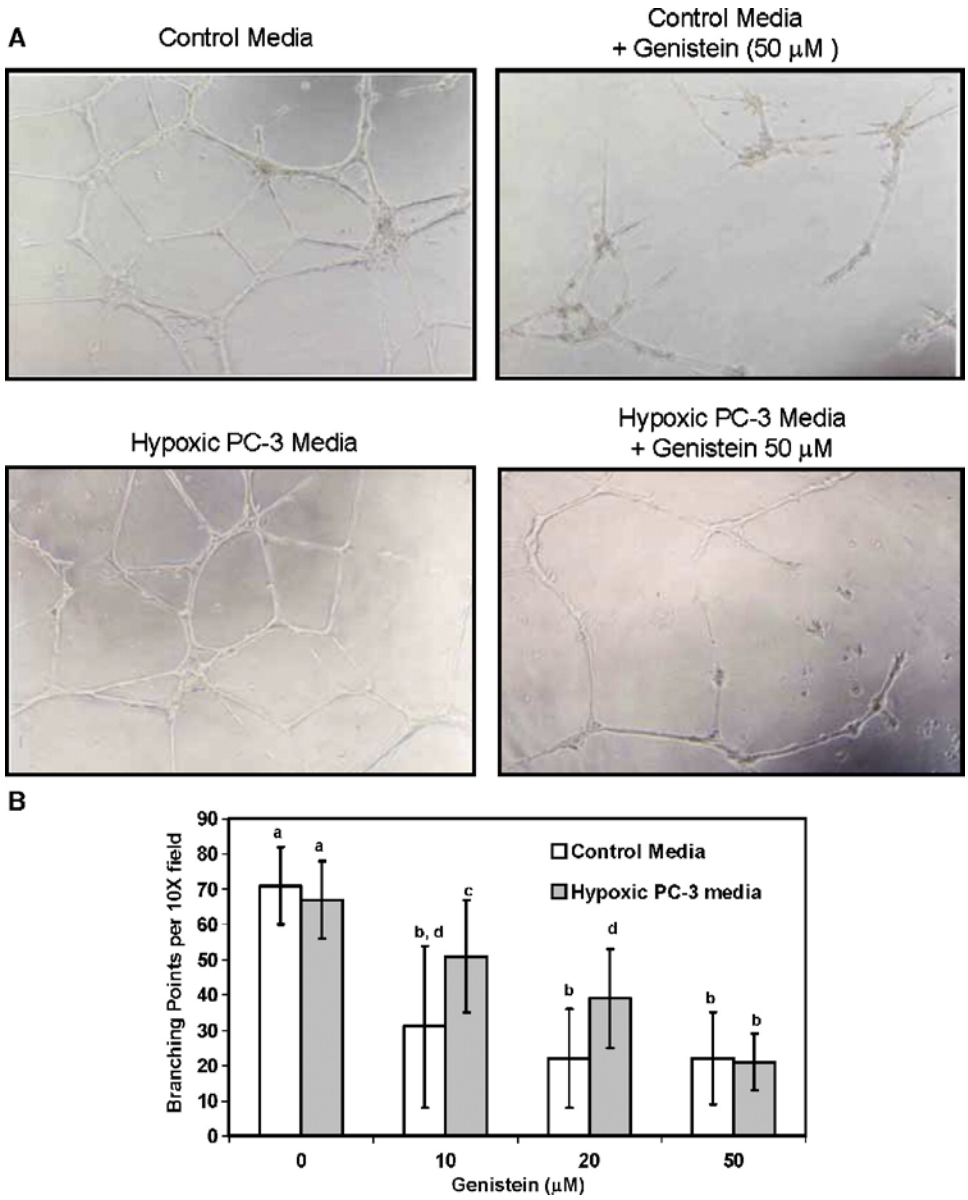


Fig. 2. Genistein reduced in vitro HUVEC tube formation in control media or in media from hypoxic prostate cancer cells. HUVECs were seeded in 96-well matrix-bottomed plates, and tube formation was evaluated by an in vitro angiogenesis assay, as described as in Materials and Methods ($n=3$). (A) Representative images showing tube formation under designated treatments. (B) Analysis of tube formation is based upon the quantitation of branching points. Error bars indicate S.D. Means with different letters indicate a statistically significant difference ($P<.05$, by ANOVA and pairwise comparison).

### 2.7. IHC staining

Cells were pretreated with vehicle or genistein for 72 h in normoxic conditions and then further incubated in a hypoxic (1% $O_2$) atmosphere for 24 h. Adherent and nonadherent cells were harvested and subsequently incorporated into a clot with thrombin and plasma, fixed in 10% neutral-buffered formalin, processed and embedded in paraffin. Sections were cut, mounted on slides and stained with an avidin–biotin–horseradish peroxidase (HRP) complex kit (Santa Cruz Biotech.). Briefly, nonspecific binding was blocked with 10% goat blocking serum then incubated overnight at 4°C with monoclonal mouse antihuman HIF-1α antibody at a 1:200 dilution (Novus Biologicals, Inc.). After washing with phosphate-buffered saline, slides were incubated with a biotinylated antimouse secondary antibody for 30 min and then treated with the avidin–HRP complex for another 30 min at room temperature, followed by color detection with diaminobenzidine and counterstaining with hematoxylin. Three representative areas were selected from each slide, and both HIF-1α-positive cells and total tumor cells were counted at a 400-fold magnification. HIF-1α staining is expressed as the percentage of positive stained cells relative to the number of total cells.

### 2.8. Statistical analysis

Data are presented as mean and S.D. The data were initially evaluated by analysis of variance (ANOVA; square root transformation was conducted as necessary) followed by Fisher's protected least significant difference test to evaluate pairwise comparisons among all treatment groups using Statview 4.5 software (Abacus Concepts, Berkeley, CA). The number of samples or replicates for each study is detailed in figure legends and in the Results section. $P<.05$ is considered significant.

## 3. Results

### 3.1. Genistein inhibits the growth of HUVECs with or without VEGF stimulation

We first measured the ability of genistein to directly modulate the in vitro growth of endothelial cells incubated in the presence or absence of VEGF. MTS assay data showed that genistein treatment at 10, 20 and 50 μM inhibited the growth of HUVECs by 25% ($P<.01$), 55% ($P<.0001$) and 69% ($P<.0001$), respectively (Fig. 1A). HUVEC growth, with the addition of 10 ng/ml supplemental VEGF, significantly increased to 138±8% ($P<.01$) compared to vehicle controls (Fig. 1A). The treatment of VEGF-supplemented HUVECs with genistein similarly produced growth inhibition. Genistein treatment at 5, 10, 20 and 50 μM inhibited VEGF-stimulated HUVEC growth by 17% ($P<.05$), 22% ($P<.01$), 51% ($P<.001$) and 71% ($P<.0001$), respectively (Fig. 1A). Thus, genistein demonstrated inhibition of HUVEC growth with an estimated $IC_{50}$ below 20 μM. These findings were confirmed by studies with direct cell counting (data not shown).

### 3.2. Genistein inhibits the proliferation of HUVECs in response to paracrine stimulation by prostate cancer cells

To examine whether prostate cancer cells may potentially induce paracrine stimulation of endothelial cell growth, we incubated HUVECs with conditioned media derived from PC-3 cells incubated for 24 h under conditions of normoxia ($pO_2=20\%$) or hypoxia ($pO_2=1\%$). As shown in Fig. 1B, media from hypoxic PC-3 cells stimulated the proliferation of HUVECs by an additional 28% ($P<.01$) compared to media derived from PC-3 cells grown under normoxic conditions. Treatment with genistein produced significant inhibition of HUVEC growth. For HUVECs treated with conditioned media from hypoxic PC-3 cells, genistein at 10, 20, 50 and 100 μM significantly inhibited growth by 34% ($P<.001$), 54% ($P<.0001$), 70% ($P<.0001$) and 72% ($P<.0001$),

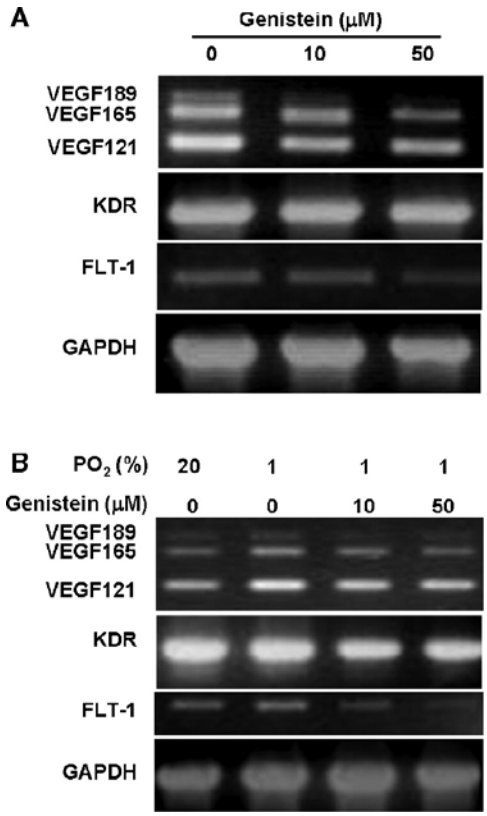


Fig. 3. Genistein modulated the expression of VEGF and VEGF receptor (KDR and FLT-1) mRNA in HUVECs under normoxic and hypoxic conditions. HUVECs were treated with designated concentrations of genistein for 72 h, and total RNA was isolated and analyzed by RT-PCR ($n=3$). The figure is a representative gel. (A) Genistein caused inhibition of VEGF mRNA expression but not of FLT-1 and KDR mRNA expression in HUVECs under normoxic conditions. (B) The mRNA expression of both VEGF and FLT-1 in HUVECs was enhanced by hypoxic exposure, which was dose-dependently inhibited by genistein. No significant change in mRNA was observed for the *KDR* gene (by ANOVA and pairwise comparison).

respectively. These findings were confirmed by studies with direct cell counting (data not shown).

The ability of HUVECs to form tubular structures in matrix-coated 96-well plates provides an in vitro system that quantitates the effects of proangiogenic and antiangiogenic factors. We observed that HUVECs formed tubular structures when incubated with either control media or media derived from PC-3 cells grown under hypoxic conditions. Tubular formation was inhibited when the cells were incubated with genistein; representative images are shown in Fig. 2A. We quantified HUVEC differentiation and tube formation by counting the number of branching points [24]. Under the conditions of our study, exposure to hypoxic conditioned media from PC-3 cells did not significantly alter the number of branching points (67±11) compared to controls (71±11) ($P>.05$). However, the addition of 10 μM genistein to control media significantly reduced the number of branching points to 31±23 ($P<.0001$, compared to vehicle), and higher concentrations (20 or 50 μM) provided no additional reduction in the number of branching points. When grown in conditioned media from hypoxic PC-3 cells, genistein at 10, 20 and 50 μM reduced the number of branching points from 67±11 to 51±16 ($P<.01$), 39±14 ($P<.0001$) and 21±8 ($P<.0001$), respectively (Fig. 2B).

### 3.3. Genistein modulates the mRNA expression of VEGF and VEGF receptors (FLT-1 and KDR) in HUVECs under normoxic and hypoxic conditions

To investigate whether genistein modulated VEGF-mediated autocrine effects on endothelial cells, we examined VEGF, FLT-1 and KDR mRNA expression in HUVECs treated with genistein. The VEGF165 isoform, which encodes a biologically active and secreted protein, was used to represent VEGF mRNA expression in response to genistein treatment. Genistein significantly decreased baseline VEGF165 mRNA expression but caused no significant changes in FLT-1 and KDR mRNA expression under normoxic conditions. A representative blot is shown in Fig. 3A. Densitometric analysis ($n=3$) showed that genistein at 10 and 50 μM inhibited VEGF165 expression by 28% ($P<.01$) and 41% ($P<.001$), respectively, compared to vehicle-treated cells.

VEGF165, FLT-1 and KDR mRNA expression in HUVECs was further examined by exposing HUVECs to genistein under hypoxic conditions (1% $O_2$). Hypoxia up-regulated VEGF and FLT-1 mRNA expression, whereas genistein (10 and 50 μM) suppressed the induction of

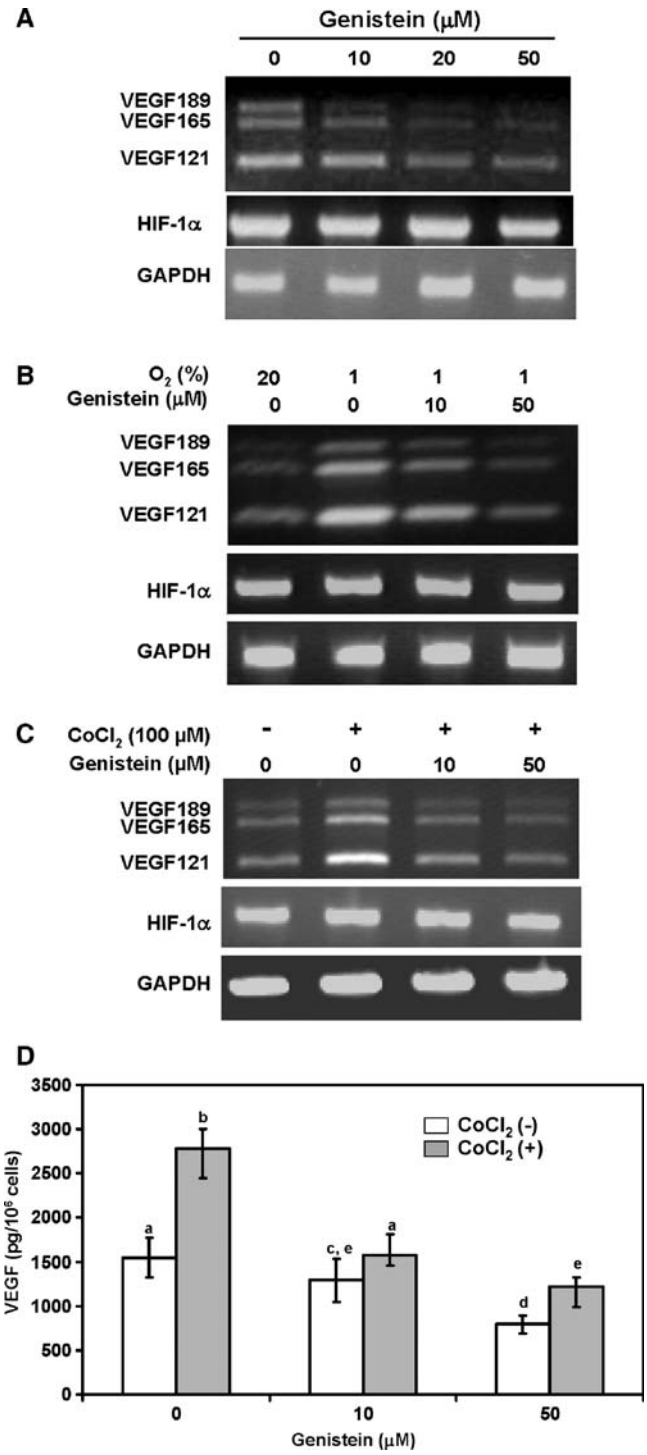


Fig. 4. Genistein modulated VEGF and HIF-1α expression in PC-3 cells under normoxic and hypoxic conditions. (A) Genistein reduced the mRNA expression of VEGF, but not of HIF-1α, in PC-3 cells under normoxic conditions. PC-3 cells were treated with genistein at designated concentrations for 72 h. Total RNA was extracted and mRNA expression was quantified after RT-PCR ($n=3$). (B) Genistein reduced the mRNA expression of VEGF, but not of HIF-1α, in PC-3 cells under hypoxic conditions. PC-3 cells were exposed to 1% $O_2$ for 24 h with or without genistein at 10 and 50 μM. Total RNA was isolated and mRNA expression was analyzed after RT-PCR ($n=3$). Hypoxia exposure induced VEGF mRNA expression in PC-3 cells. (C) Genistein inhibited the $CoCl_2$-induced expression of VEGF mRNA in PC-3 cells. PC-3 cells were exposed to $CoCl_2$ (100 μM) for 24 h with or without genistein at 10 and 50 μM. Total RNA was isolated and mRNA expression was analyzed after RT-PCR ($n=3$). (D) Genistein inhibited VEGF secretion (ELISA) in PC-3 cells incubated with or without $CoCl_2$ ($n=4$). Cells were exposed to 10 or 50 μM genistein with or without $CoCl_2$ (100 μM) for 48 h. Data are presented as mean±S.D. Means with different letters indicate a statistically significant difference ($P<.05$, by ANOVA and pairwise comparison).

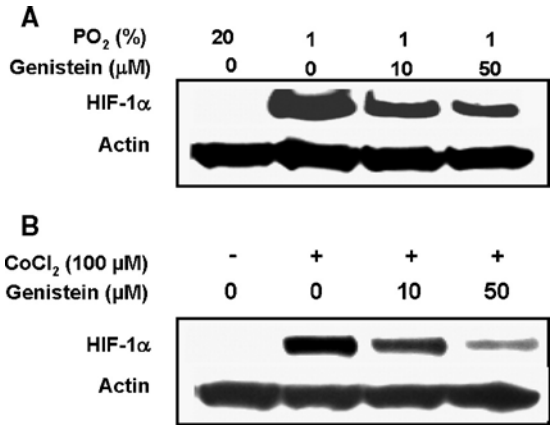


Fig. 5. (A) Genistein inhibited hypoxia-induced HIF-1α protein expression in PC-3 cells. Cells were exposed to 1% $O_2$ for 24 h with or without 24-h genistein pretreatment (10 and 50 μM). Nuclear extracts were prepared for Western blot analysis ($n=3$). Hypoxia exposure stabilized HIF-1α protein in PC-3 cells, and genistein inhibited HIF-1α induction in response to hypoxia. (B) Genistein inhibited $CoCl_2$ (100 μM)-induced HIF-1α protein expression in PC-3 cells. Cells were first exposed to vehicle or designated genistein for 24 h and then were further incubated with $CoCl_2$ (100 μM) for another 24 h. Nuclear extracts were prepared for Western blot analysis ($n=3$). $CoCl_2$ (100 μM) exposure increased HIF-1α protein in PC-3 cells, which was inhibited by genistein treatment.

VEGF165 and FLT-1 mRNA expression (Fig. 3B). Densitometric analysis ($n=3$) showed that 24-h exposure to 1% $O_2$ induced a 2.4-fold ($P<.001$) and a 2.3-fold ($P<.001$) increase in VEGF and FLT-1 mRNA expression in HUVECs, respectively. Genistein at 10 and 50 μM also inhibited hypoxia-induced VEGF165 mRNA expression by 21% ($P<.01$) and 28% ($P<.01$), respectively. Treatment with genistein at 10 and 50 μM showed inhibition of FLT-1 mRNA expression by 51% ($P<.001$) and 70% ($P<.001$), respectively. A slight decrease without statistically significant changes in KDR mRNA expression was observed in HUVECs under hypoxic conditions with or without exposure to genistein.

### *3.4. Genistein modulates VEGF expression in PC-3 cells under normoxic and hypoxic conditions*

To determine whether genistein and hypoxia interact to modulate VEGF expression, we examined VEGF165 mRNA and protein expression in PC-3 cells under normoxic and hypoxic conditions. As shown in Fig. 4A, genistein down-regulated VEGF165 mRNA expression in PC-3 cells under normoxic conditions. Densitometric analyses ($n=3$) of RT-PCR results showed that, compared to vehicle-treated PC-3 cells (density=1.00), the densities of VEGF165 mRNA from genistein-treated cells are 0.74 at 10 μM ($P<.01$), 0.65 at 20 μM ($P<.01$) and 0.54 at 50 μM ($P<.001$). Genistein modulation of PC-3 VEGF gene expression was further studied under hypoxic conditions. Fig. 4B demonstrates that exposure to 1% $O_2$ significantly up-regulated VEGF mRNA expression and that genistein countered this effect. Further densitometric analysis showed that, compared to control (density=1.00), 1% $O_2$ incubation for 24 h induced a 4.6-fold increase ($P<.0001$) in VEGF165 mRNA expression, and 10 and 50 μM genistein inhibited hypoxia-induced VEGF165 mRNA expression by 27% ($P<.01$) and 51% ($P<.001$), respectively. $CoCl_2$, an established hypoxia mimetic agent, showed similar results, as seen with chamber-induced hypoxia with regard to VEGF expression (Fig. 4C). Densitometric analysis showed that, compared to controls (density=1.00), $CoCl_2$ (100 μM) incubation for 24 h induced a 2.3-fold increase ($P<.001$) in PC-3 VEGF165 mRNA expression, and 10 and 50 μM genistein inhibited $CoCl_2$-induced VEGF165 mRNA expression by 44% ($P<.001$) and 61% ($P<.0001$), respectively. We subsequently examined VEGF secretion by PC-3 cells under similar conditions. PC-3 cells showed a basal secretion of VEGF protein at $1548\pm224$ pg/$10^6$ cells after incubation in normoxic conditions for 48 h (Fig. 4D). Genistein at 10 and 50 μM inhibited PC-3 VEGF secretion under normoxic conditions by 17% ($P<.05$) and 49% ($P<.001$), respectively. Exposure of PC-3 cells to the hypoxic mimetic agent $CoCl_2$ (100 μM) induced a significant increase in VEGF protein secretion by 80% ($P<.001$, compared to vehicle control). Genistein at 10 μM reduced $CoCl_2$-induced VEGF secretion by 44% ($P<.001$) compared to cells without genistein treatment. Exposure to 50 μM genistein in $CoCl_2$ incubation conditions further decreased VEGF protein secretion by 56% ($P<.001$, compared to vehicle).

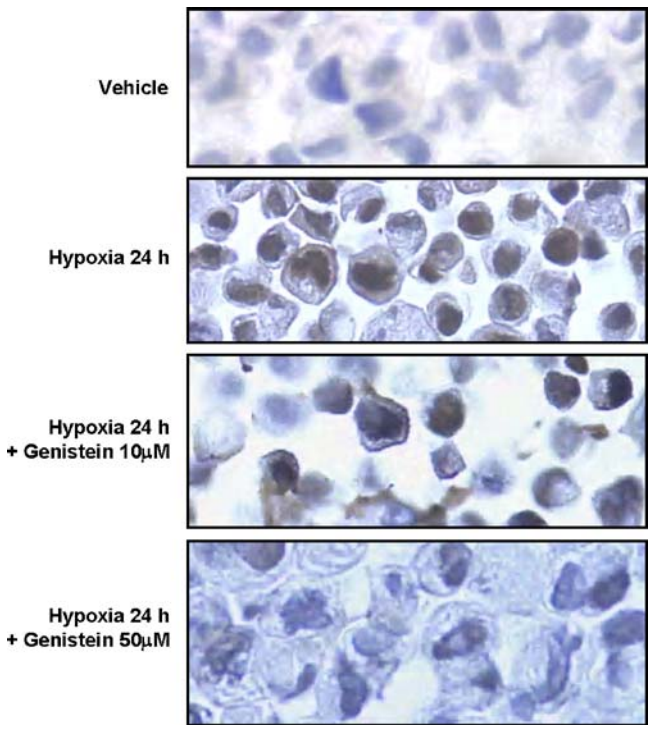


Fig. 6. IHC staining showed the inhibition of hypoxia-induced HIF-1α protein expression in PC-3 cells by genistein at 10 and 50 μM. Cells were exposed to 1% $O_2$ for 24 h with or without 10 and 50 μM genistein 24 h pretreatment. HIF-1α expression was examined by IHC staining ($n=3$). Hypoxia exposure caused an increase in the nuclear staining of HIF-1α.

### 3.5. Genistein modulates HIF-1α expression in PC-3 cells under normoxic and hypoxic conditions

To gain insight into the molecular mechanisms by which genistein inhibits hypoxia-induced VEGF expression, the expression of HIF-1α mRNA and protein was further investigated in genistein-treated PC-3 cells under normoxic and hypoxic conditions. Genistein did not alter the mRNA expression of HIF-1α in PC-3 cells under normoxic conditions (Fig. 4A). Hypoxia exposure did not cause a significant change in HIF-1α mRNA expression, and treatment with genistein (10 and 50 μM) had no effect on HIF-1α mRNA expression in PC-3 cells (Fig. 4B). However, Western blot analysis showed no detectable nuclear HIF-1α protein in normoxic PC-3 cells. Hypoxia-exposed PC-3 cells showed a dramatic increase in HIF-1α protein expression (Fig. 5A). Densitometric analysis showed that, compared to vehicle, genistein treatment caused a dose-dependent reduction in hypoxia-induced nuclear HIF-1α content at 10 μM by 36% ($P<.01$) and at 50 μM by 54% ($P<.001$) (Fig. 5A). $CoCl_2$ showed results similar to those of gas-chamber-induced hypoxia in the induction of HIF-1α protein expression. PC-3 cells incubated with $CoCl_2$ at 100 μM showed a dramatic increase in HIF-1α protein expression (Fig. 5A). Densitometric analysis showed that, compared to vehicle, genistein treatment caused a dose-dependent reduction in $CoCl_2$-induced nuclear HIF-1α expression at 10 μM by 47% ($P<.01$) and at 50 μM by 74% ($P<.001$) (Fig. 5A). Consistent with Western blot analysis results, IHC staining showed a significant increase in the nuclear staining of HIF-1α in PC-3 cells exposed to hypoxia (87%) compared to vehicle ($P<.0001$) (Fig. 6). Treatment with genistein showed a decrease in nuclear staining for HIF-1α in PC-3 cells exposed to hypoxia. In cells treated with 10 and 50 μM genistein, only 56% and 25% of cells showed positive nuclear staining for HIF-1α, respectively (both $P<.01$, compared to hypoxia controls). These results are consistent with posttranscriptional regulation of HIF-1α by both hypoxia and genistein.

## 4. Discussion

Angiogenesis is hypothesized to be indispensable for the development and progression of prostate cancer. Tumor angiogenesis is dependent upon complex autocrine and paracrine interactions between malignant tumor cells and components of the tumor microenvironment that include matrix and vascular cells, as well as modulators derived from the circulation [1,7]. VEGF has emerged as one of the critical proangiogenic growth factors associated with prostate carcinogenesis [15–20]. Inhibitors of specific steps in the angiogenesis cascade, including VEGF production and signaling, are now showing success in clinical trials as therapeutic agents for malignancies, including prostate cancer [25,26] We propose that tumor angiogenesis may also serve as a target for dietary interventions or chemopreventive agents for high-risk populations to significantly delay the development of prostate cancer or to even prevent clinically significant diseases.

In support of this concept, soy products have been shown to reduce prostate tumor growth in rodent models [23,27]. We demonstrated that feeding isoflavone-rich soy protein or soy phytochemical concentrate significantly reduced microvessel density in murine transplanted prostate tumors [23]. We observed that microvessel density was reduced by 30–40% in mice fed soy components, with a 50–100% increase in apoptosis and a modest reduction in proliferation of about 10–15% [23]. Interestingly, a similar pattern of biomarker changes is observed when antiangiogenic agents are administered in murine models of cancer, suggesting that soy components may share some biological activity with antiangiogenic agents. These findings regarding prostate cancer are mutually supportive of several key published reports from studies of cancers derived from other tissues. Daily genistein (0.5 mg/kg) injections reduced microvessel density in murine xenografted human HSC-3 oral squamous cell carcinoma [28]. Genistein administered by injection has been shown to reduce the tumor blood supply of mice transplanted with Lewis lung cancer cells and B16 melanoma cells [29].

Current studies using in vitro systems provide insight into biologically plausible mechanisms that could underlie the above documented in vivo observations. We observed that soy isoflavones cause inhibition of (a) VEGF-mediated endothelial cell proliferation and tube formation; (b) hypoxia-induced VEGF expression in both prostate cancer cells and endothelial cells; (c) VEGF receptor expression by endothelial cells; and (d) HIF-1α expression by prostate cancer cells in response to hypoxia. Taken together, these observations suggest that soy isoflavones may act at several autocrine and paracrine steps in VEGF-mediated prostate tumor angiogenesis. The production of VEGF in response to hypoxia is blunted, in part, due to a reduction in HIF-1α. Subsequent VEGF bioactivity in vascular endothelial cells (proliferation and differentiation) is suppressed by isoflavones, probably through both inhibition of VEGF receptor expression and blocking of receptor-mediated tyrosine kinase activity, as well as downstream signaling pathways [30]. Our findings with prostate cancer cells are supported by others who have shown that genistein inhibits hypoxia-induced VEGF mRNA expression in kidney 293 cells, U87 glioma cells, HT1080 human fibrosarcoma cells [31], skin keratinocytes [32], bladder cancer cells [33] and renal cell carcinoma cells [34]. A recent rodent study showed that genistein (0.5 mg/kg) injections reduced VEGF mRNA expression in transplanted oral squamous cell carcinoma in mice [28]. Fotsis et al. [35] demonstrated that urine fractions from humans consuming a soy-rich plant-based diet contains genistein and other isoflavonoids that inhibit bovine-brain-derived capillary endothelial cell proliferation in vitro.

Although in vitro data support in vivo rodent findings, questions on dose–response relationships and the concen-

tration of isoflavones necessary to achieve specific outcomes remain areas of frequent debate. For example, our in vitro studies suggest that the disruption of VEGF autocrine and paracrine signaling can be observed in the range of 10–25 μM genistein. It is well known that blood concentrations of soy isoflavones, over the range of typical human and rodent consumption, are typically <10 μM and are quickly cleared from circulation. Reconciliation of this apparent inconsistency will require additional research, but if we consider the striking differences between the optimized microenvironment of cells in culture and those within a growing tumor, some hypotheses emerge. The intratumor environment is characterized by hypoxia, relatively high interstitial pressure, poor perfusion and diffusion, leading to a lack of critical nutrients and the accumulation of metabolic waste. It is likely that the harsh in vivo environment places tumor cells at a lower threshold for the activation of apoptotic cascades, just like serum starvation is often used to enhance in vitro sensitivity to phytochemicals, essentially lowering the minimally effective concentration.

We focused our efforts on VEGF, while it remains to be seen how other key processes in the prostate tumor microenvironment may also be influenced by soy isoflavones to alter the finely orchestrated balance between multiple proangiogenic and antiangiogenic regulators. A recent publication [33] from the bladder cancer literature provides insight into the ability of soy isoflavones to inhibit the expression of proangiogenesis activators, including urokinase plasminogen activator and matrix metalloprotease enzymes, by transitional cell carcinoma cells while stimulating the expression of antiangiogenic genes such as *thrombospondin-1*, *endostatin* and *angiostatin*.

We examined HIF-1α due, in part, to its critical role in regulating VEGF expression and our desire to determine whether genistein acts partially through this pathway, but also due to the anticipation that HIF-1α may serve as a candidate biomarker of soy activity for future rodent or human studies. The regulation of HIF-1α content in tumor cells appears to be primarily due to posttranslational mechanisms. HIF-1α protein is constitutively synthesized and rapidly degraded by the ubiquitin–proteasome pathway under normoxic conditions. However, the cellular content of HIF-1α is dramatically increased by hypoxia, probably through a combination of increased synthesis and decreased degradation [12,13,36]. HIF-1α has been shown to enhance VEGF expression in LNCaP cells [37]. We demonstrated that hypoxia significantly increased the levels of HIF-1α in parallel with an increased expression of VEGF in PC-3 cells. The addition of genistein caused a decrease in hypoxia-enhanced HIF-1α protein expression in PC-3 cells. Others have also suggested a similar effect of isoflavones on HIF-1α-regulated processes in pancreatic carcinoma cells [38] and Hep3B hepatoma cells [39]. Previous studies have suggested that the phosphatidylinositol 3′-kinase (PI3K) pathway is essential for the stabilization of HIF-1α protein [40]. Data from our laboratory, as well as from other groups, have shown that genistein can cause inhibition of PI3K/Akt activation in prostate cancer calls and other cell lines [41]. Thus, inhibition of the PI3K pathway may be one of the mechanisms contributing to the effect of genistein on HIF-1α.

In addition to the regulation of VEGF and HIF-1α expression, our current studies show that genistein may inhibit angiogenesis by reducing receptor content in endothelial cells. We observed an enhanced expression of FLT-1 mRNA in HUVECs exposed to hypoxia and inhibition by genistein exposure. VEGF treatment has been shown to induce the tyrosyl phosphorylation of KDR/Flk-1 and the activation of p44/p42 MAPK and AKT pathways [42–45], implicating that signal transduction plays an important role in mediating VEGF function. Previous studies in our laboratory have shown that genistein inhibits the phosphorylation of tyrosyl kinase, Akt and p44/p42 MAPK proteins in prostate cancer [41]. These observations suggest that the regulation of VEGF receptor levels and downstream intracellular signaling pathways contributes to the antiangiogenic effects of genistein.

In summary, we report that genistein may influence prostate cancer angiogenesis via several autocrine and paracrine mechanisms. Genistein reduces VEGF production in prostate cancer cells with or without the presence of hypoxia. In addition, genistein targets endothelial cells by reducing VEGF and VEGF receptor expression. These observations may, in part, be associated with a change in the HIF-1α regulation of VEGF expression. Thus, additional efforts to characterize how soy isoflavones may alter autocrine and paracrine growth factor networks associated with angiogenesis are warranted.

## References

[1] Folkman J. Fundamental concepts of the angiogenic process. Curr Mol Med 2003;3(7):643–51.
[2] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57–70.
[3] van der Bilt JD, Borel Rinkes IH. Surgery and angiogenesis. Biochim Biophys Acta 2004;1654(1):95–104.
[4] Tonini T, Rossi F, Claudio PP. Molecular basis of angiogenesis and cancer. Oncogene 2003;22(42):6549–56.
[5] Ferrara N. The role of VEGF in the regulation of physiological and pathological angiogenesis. Exs 2005;(94):209–31.
[6] Bikfalvi A, Sauzeau C, Moukadiri H, Maclouf J, Busso N, Bryckaert M, et al. Interaction of vasculotropin/vascular endothelial cell growth factor with human umbilical vein endothelial cells: binding, internalization, degradation, and biological effects. J Cell Physiol 1991;149(1):50–9.
[7] Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3(6):401–10.
[8] Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A 1995;92(8):3566–70.
[9] Zachary I. Vascular endothelial growth factor: how it transmits its signal. Exp Nephrol 1998;6(6):480–7.
[10] Richard DE, Berra E, Pouyssegur J. Angiogenesis: how a tumor adapts to hypoxia. Biochem Biophys Res Commun 1999;266(3):718–22.

[11] Brown JM. Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. Mol Med Today 2000;6(4):157–62.
[12] Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an $O_2$-dependent degradation domain via the ubiquitin–proteasome pathway. Proc Natl Acad Sci U S A 1998; 95(14):7987–92.
[13] Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 2000;88(4):1474–80.
[14] Semenza GL. HIF-1: using two hands to flip the angiogenic switch. Cancer Metastasis Rev 2000;19(1–2):59–65.
[15] Brawer MK, Deering RE, Brown M, Preston SD, Bigler SA. Predictors of pathologic stage in prostatic carcinoma. The role of neovascularity. Cancer 1994;73(3):678–87.
[16] Liao Z, Boileau TW, Erdman Jr JW, Clinton SK. Interrelationships among angiogenesis, proliferation, and apoptosis in the tumor microenvironment during *N*-methyl-*N*-nitrosourea androgen-induced prostate carcinogenesis in rats. Carcinogenesis 2002;23(10): 1701–11.
[17] Mazzucchelli R, Montironi R, Santinelli A, Lucarini G, Pugnaloni A, Biagini G. Vascular endothelial growth factor expression and capillary architecture in high-grade PIN and prostate cancer in untreated and androgen-ablated patients. Prostate 2000;45(1):72–9.
[18] Zhong H, Semenza GL, Simons JW, De Marzo AM. Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis. Cancer Detect Prev 2004;28(2):88–93.
[19] Shariat SF, Anwuri VA, Lamb DJ, Shah NV, Wheeler TM, Slawin KM. Association of preoperative plasma levels of vascular endothelial growth factor and soluble vascular cell adhesion molecule-1 with lymph node status and biochemical progression after radical prostatectomy. J Clin Oncol 2004;22(9):1655–63.
[20] George DJ, Halabi S, Shepard TF, Vogelzang NJ, Hayes DF, Small EJ, et al. Prognostic significance of plasma vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B 9480. Clin Cancer Res 2001;7(7):1932–6.
[21] Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun 2005;333(2):328–35.
[22] Mukherjee P, Sotnikov AV, Mangian HJ, Zhou JR, Visek WJ, Clinton SK. Energy intake and prostate tumor growth, angiogenesis, and vascular endothelial growth factor expression. J Natl Cancer Inst 1999;91(6):512–23.
[23] Zhou JR, Gugger ET, Tanaka T, Guo Y, Blackburn GL, Clinton SK. Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. J Nutr 1999; 129(9):1628–35.
[24] Kim HJ, Vosseler CA, Weber PC, Erl W. Docosahexaenoic acid induces apoptosis in proliferating human endothelial cells. J Cell Physiol 2005;204(3):881–8.
[25] Pfeffer U, Ferrari N, Morini M, Benelli R, Noonan DM, Albini A. Antiangiogenic activity of chemopreventive drugs. Int J Biol Markers 2003;18(1):70–4.
[26] Carroll PR, Chan JM, D'Amico AV, Gelmann EP, Iversen P, Klotz L, et al. Fourth International Conference on Innovations and Challenges in Prostate Cancer: Prevention, Detection and Treatment. J Urol 2004; 172(5 Pt 2):S3–S35.
[27] Aronson WJ, Tymchuk CN, Elashoff RM, McBride WH, McLean C, Wang H, et al. Decreased growth of human prostate LNCaP tumors in SCID mice fed a low-fat, soy protein diet with isoflavones. Nutr Cancer 1999;35(2):130–6.
[28] Myoung H, Hong SP, Yun PY, Lee JH, Kim MJ. Anti-cancer effect of genistein in oral squamous cell carcinoma with respect to angiogenesis and in vitro invasion. Cancer Sci 2003;94(2):215–20.
[29] Wietrzyk J, Boratynski J, Grynkiewicz G, Ryczynski A, Radzikowski C, Opolski A. Antiangiogenic and antitumour effects in vivo of genistein applied alone or combined with cyclophosphamide. Anticancer Research 2001;21(6A):3893–6.
[30] Simons M. Integrative signaling in angiogenesis. Mol Cell Biochem 2004;264(1–2):99–102.
[31] Mukhopadhyay D, Tsiokas L, Sukhatme VP. High cell density induces vascular endothelial growth factor expression via protein tyrosine phosphorylation. Gene Expr 1998;7(1):53–60.
[32] Trompezinski S, Denis A, Schmitt D, Viac J. Comparative effects of polyphenols from green tea (EGCG) and soybean (genistein) on VEGF and IL-8 release from normal human keratinocytes stimulated with the proinflammatory cytokine TNFalpha. Arch Dermatol Res 2003;295(3):112–6.
[33] Su SJ, Yeh TM, Chuang WJ, Ho CL, Chang KL, Cheng HL, et al. The novel targets for anti-angiogenesis of genistein on human cancer cells. Biochem Pharmacol 2005;69(2):307–18.
[34] Sasamura H, Takahashi A, Miyao N, Yanase M, Masumori N, Kitamura H, et al. Inhibitory effect on expression of angiogenic factors by antiangiogenic agents in renal cell carcinoma. Br J Cancer 2002;86(5):768–73.
[35] Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L. Genistein, a dietary-derived inhibitor of in vitro angiogenesis, Proceedings of the National Academy of Sciences of the United States of America. 1993;90(7):2690–94
[36] Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin–proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997;272(36):22642–7.
[37] Mabjeesh NJ, Willard MT, Frederickson CE, Zhong H, Simons JW. Androgens stimulate hypoxia-inducible factor 1 activation via autocrine loop of tyrosine kinase receptor/phosphatidylinositol 3′-kinase/protein kinase B in prostate cancer cells. Clin Cancer Res 2003; 9(7):2416–25.
[38] Buchler P, Reber HA, Buchler MW, Friess H, Lavey RS, Hines OJ. Antiangiogenic activity of genistein in pancreatic carcinoma cells is mediated by the inhibition of hypoxia-inducible factor-1 and the down-regulation of VEGF gene expression. Cancer 2004;100(1): 201–10.
[39] Wang GL, Jiang BH, Semenza GL. Effect of protein kinase and phosphatase inhibitors on expression of hypoxia-inducible factor 1. Biochem Biophys Res Commun 1995;216(2):669–75.
[40] Sandau KB, Faus HG, Brune B. Induction of hypoxia-inducible-factor 1 by nitric oxide is mediated via the PI 3K pathway. Biochem Biophys Res Commun 2000;278(1):263–7.
[41] Wang S, DeGroff VL, Clinton SK. Tomato and soy polyphenols reduce insulin-like growth factor-I-stimulated rat prostate cancer cell proliferation and apoptotic resistance in vitro via inhibition of intracellular signaling pathways involving tyrosine kinase. J Nutr 2003;133(7):2367–76.
[42] Murata M, Kador PF, Sato S. Vascular endothelial growth factor (VEGF) enhances the expression of receptors and activates mitogen-activated protein (MAP) kinase of dog retinal capillary endothelial cells. J Ocul Pharmacol Ther 2000;16(4):383–91.
[43] Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, et al. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;60(6):1541–5.
[44] Takahashi N, Seko Y, Noiri E, Tobe K, Kadowaki T, Sabe H, et al. Vascular endothelial growth factor induces activation and subcellular translocation of focal adhesion kinase (p125FAK) in cultured rat cardiac myocytes. Circ Res 1999;84(10):1194–202.
[45] Guo D, Jia Q, Song HY, Warren RS, Donner DB. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. J Biol Chem 1995;270(12):6729–33.